

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



A19

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 31/365, C07D 307/33		A1	(11) International Publication Number: WO 97/27851
			(43) International Publication Date: 7 August 1997 (07.08.97)
<p>(21) International Application Number: PCT/US97/01241</p> <p>(22) International Filing Date: 31 January 1997 (31.01.97)</p> <p>(30) Priority Data: 60/010,996 1 February 1996 (01.02.96) US</p> <p>(71) Applicant: THE JOHNS-HOPKINS UNIVERSITY [US/US]; 3400 N. Charles Street, Baltimore, MD 21818 (US).</p> <p>(72) Inventors: BISHAI, William, R.; 900 East 37th Street, Baltimore, MD 21218 (US). CHEN, Ping; 1118 Bellemore Road, Baltimore, MD 21210 (US). DICK, James, D.; 3512 Cornwall Court, Baltimore, MD 21222 (US). PARRISH, Nikki; Apartment 201, 3185 Pine Orchard Lane, Ellicott City, MD 21042 (US).</p> <p>(74) Agents: KOKULIS, Paul, N. et al.; Cushman Darby & Cushman, Intellectual Property Group of Pillsbury Madison & Sutro, 1100 New York Avenue, N.W., Washington, DC 20005 (US).</p>			<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>
<p>(54) Title: L-HOMOSERINE AND L-HOMOSERINE LACTONE AS BACTERIOSTATIC AGENTS FOR M. TUBERCULOSIS AND M. BOVIS</p> <p>(57) Abstract</p> <p>A method for inhibiting growth of slow growing mycobacteria involves administering an inhibitory amount of L-homoserine, L-homoserine lactone or a precursor thereof to mycobacteria. This method can be used to detect the presence of slow growing mycobacteria and to treat patients infected with <i>M. tuberculosis</i>.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LI	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroun	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

**L-HOMOSERINE AND L-HOMOSERINE LACTONE
AS BACTERIOSTATIC AGENTS FOR
M. TUBERCULOSIS AND *M. BOVIS***

BACKGROUND OF THE INVENTION

5 Several mycobacteria are important human pathogens and establish persistent infections. *M. tuberculosis* and *M. bovis* are two species of mycobacteria that cause tuberculosis. *M. tuberculosis* is resistant to the enzyme activities of neutrophils and/or macrophages. As a result, *M. tuberculosis* survives, continuing to grow and later to cause infection because of the failure of these phagocytic cells to kill the invading pathogens.

10 Treatment of tuberculosis is based upon intensive and prolonged exposure of the organisms to bacterial antagonists such as antibiotics. However, drug resistance is a significant problem. Drug resistance in tuberculosis is time consuming to diagnose and is a leading cause of morbidity from the infection. In New York, the incidence of multidrug resistant tuberculosis (MDRTB) reached 30% in 1991 (Frieden et al., N. Eng. J. Med., 328:521-526, 1993). About 80% of MDRTB cases occur in HIV-infected individuals, and the combination of HIV and MDRTB has been reported to carry a mortality of 70-89% (Fischl et al., Ann. Intern. Med., 117:177-183, 1992; Fischl et al., Ann. Intern. Med., 117:184-190, 1992; Dooley et al., Ann. Intern. Med., 117:257-259, 1992). The last new drug for tuberculosis, rifampin, was introduced in 1967 (Wolinsky, In: Gorbach et al., eds. *Infectious Diseases*. W.B. Saunders, Philadelphia, pp. 313-319, 1994). In spite of the numerous unique aspects of mycobacterial cellular physiology which might provide novel drug targets,

only recently has interest been renewed in identifying such targets.

The only absolute proof of active tuberculosis disease is the culture and identification of *M. tuberculosis* from tissue or body fluid (see for example, the description in U.S. Pat. No. 5,582,985). Most clinical mycobacteriology laboratories currently use radiometric liquid culture systems (e.g., the Bactec 460 TB machine, Becton-Dickinson, Sparks, MD) in combination with DNA probes (e.g., AccuProbe Kit, Gen-Probe, Inc., San Diego, CA) for species identification. Radiometric detection with liquid culture yields positive cultures in an average of 10-15 days.

However, after acid-fast bacilli (AFB) are detected, there is a waiting period of 3-6 days before enough bacilli have grown to perform the DNA probe analysis for species identification (Miller et al., J. Clin. Microbiol., 32:393-397, 1994). Hence the entire process requires 14-21 days to complete (Heifets et al., In: Bloom B.R., ed. *Tuberculosis: Pathogenesis, Protection and Control*, ASM Press, Washington, DC, pp. 85-110, 1994).

DNA probe hybridization is the current state-of-the-art technology for species identification once an isolate has been grown up in the standard cultivation medium. The DNA probe test is expensive, difficult to perform in developing countries, and requires an additional day of testing beyond the 3-6 additional days required to grow the isolate to sufficient levels. There is a need in the art to develop new drugs to treat tuberculosis, especially that caused by multidrug resistant *M. tuberculosis*. There is also a need in the art to develop new

diagnostic tests to achieve identification inexpensively, simply, and without the need for 4-7 additional days of testing.

SUMMARY OF THE INVENTION

5 It is an object of the invention to provide methods for inhibiting growth of slow growing mycobacteria.

10 It is yet another object of the invention to provide methods for identifying slow growing mycobacteria.

15 These and other objects of the invention are provided by one or more of the embodiments described below. In one embodiment of the invention there is provided a method for inhibiting growth of slow growing mycobacteria which comprises administering to said mycobacteria an inhibiting amount of L-homoserine, L-homoserine lactone or a precursor thereof.

20 A method for identifying slow growing mycobacteria is provided by this invention. The method comprises inoculating a clinical specimen into a cultivation medium in the presence of and in the absence of L-homoserine, L-homoserine lactone or a precursor thereof. If the specimen grows in the 25 cultivation medium in the absence of these compounds, but not in the medium which contains the compounds, the presence of a slow growing mycobacterium is indicated.

Preferred slow growing mycobacteria that are 30 targets of the invention are tuberculous mycobacteria and non-tuberculous mycobacteria. Tuberculous mycobacteria include *M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*; non-

tuberculous mycobacteria include *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. leprae*.

Because the compounds of the present invention act by a mechanism which is different from those characterized for known anti-mycobacterial agents, a combination of the different classes of anti-mycobacterial agents provides a composition useful for inhibiting growth of slow growing mycobacteria.

10 A method for identifying targets of L-homoserine, L-homoserine lactone, or a precursor thereof that cause growth inhibition is provided by the invention.

The present invention provides the art with methods of using compounds which are specifically bacteriostatic for slow growing mycobacteria. This invention thus provides methods for identifying and treating tuberculosis, especially tuberculosis caused by multidrug resistant *M. tuberculosis*. This 20 invention also provides an inexpensive, simple and quick diagnostic test which distinguishes slow growing mycobacteria, including *M. tuberculosis* complex organisms, from non-tuberculous, rapid growing mycobacteria in the clinical laboratory. 25 Moreover, the invention provides a mycobacterial cell line resistant to growth inhibition by L-homoserine, L-homoserine lactone, or a precursor thereof useful as a control in the methods of the invention.

30 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the metabolic biosynthesis of homoserine, homoserine phosphate, and homoserine lactone. Enzymes that control each

step of the metabolic pathway are shown beneath the reaction arrows.

Figure 2 shows chemical compounds useful in understanding the present invention.

5 Figure 3 shows growth-inhibited *M. bovis* BCG exposed to L-homoserine lactone are still metabolically active as measured by continued utilization of uracil. Panel A shows treatment with 50 $\mu\text{g}/\text{ml}$, panel B shows treatment with 100 $\mu\text{g}/\text{ml}$,
10 and panel C shows treatment with 200 $\mu\text{g}/\text{ml}$ for medium alone (square), L-homoserine lactone (circle), and L-homoserine (diamond). The abscissa is time of treatment in days, and the ordinate is counts per 4 min.

15 Figure 4 shows inhibition of *M. bovis* BCG growth by homoserine as determined by uracil, acetate, or methionine uptake. D-homoserine is shown in panels A, C and E at 50 $\mu\text{g}/\text{ml}$ (diamond) or 200 $\mu\text{g}/\text{ml}$ (circle); L-homoserine is shown in panels B, D
20 and F at 50 $\mu\text{g}/\text{ml}$ (diamond) or 200 $\mu\text{g}/\text{ml}$ (circle); and controls are medium alone (square) and D-cycloserine at 50 $\mu\text{g}/\text{ml}$ (triangle). The abscissa is time in days, and the ordinate is counts per 4 min.

25 Figure 5 shows survival of *M. bovis* BCG after homoserine or homoserine lactone treatment. From top to bottom: square represents phosphate-buffered saline, diamond represents D-homoserine, circle represents L-homoserine lactone, inverted triangle represents
30 ethambutol, and asterisk represents isoniazid. The abscissa is time of treatment in days, and the ordinate is colony forming units per milliliter.

Figure 6 shows specific inhibition by L-homoserine of glutamate uptake in wild type *M. bovis*

BCG and L-homoserine resistant *M. bovis* BCG. Panel A illustrates glutamate uptake in wild type *M. bovis* BCG determined against increasing concentrations of L-homoserine (filled square) or D-homoserine (open square). The dpms obtained from the experimental samples were normalized to controls. When the value was greater than a hundred, 100% was used. Panel B glutamate uptake in the presence of 200 µg/ml L-homoserine in wild type *M. bovis* BCG as well as two L-homoserine resistant *M. bovis* BCG strains. The error bar indicates the standard deviation for each strain tested.

DETAILED DESCRIPTION OF THE INVENTION

It is a discovery of the present invention that L-homoserine and its metabolites can inhibit the growth of slow growing mycobacteria. In particular, these compounds are specifically bacteriostatic for slow growing mycobacteria including *M. tuberculosis*, multidrug resistant isolates of *M. tuberculosis*, and *M. bovis*. This discovery provides the basis for using these compounds therapeutically to alleviate clinical infections and diagnostically to identify whether a potentially pathogenic species is present.

Compounds which may be used according to the present invention include homoserine, homoserine lactone, and precursors thereof. The precursors are compounds that can be converted to L-homoserine or L-homoserine lactone under the conditions known in the art (see Figure 1). The precursors include L-homoserine phosphate.

The bacteria which are susceptible to growth inhibition by homoserine and its metabolites are

mycobacteria. Mycobacteria, are slender, straight or curved rods. They usually occur singly or in clusters but may occasionally exhibit a branching and filamentous form. Mycobacteria are well characterized in the art. They are usually acid fast and grow on ordinary medium known in the art. The time interval required for the cell to divide or for the population to double is known as the generation time. Not all bacteria have the same generation time; for some rapid growers, such as *E. coli*, it is 15 to 20 minutes; for others it is hours. Slow growing mycobacteria have a generation time of at least 10 hours; for *M. tuberculosis*, and for *M. bovis* the generation time is about 24 hours. In general, clinical mycobacteriology laboratories cannot rely upon the time it takes for a body fluid specimen to grow a mycobacterial species to determine whether the species is a rapid grower or a slow grower, because the density of the inoculum is not known. For example, a heavily infected sputum containing slow growing *M. tuberculosis* might turn positive in 7 days, while a lightly colonized sputum containing rapid growing *M. chelonae* might turn positive in 12 days. Usually slow growing mycobacteria, e.g., *M. tuberculosis*, are sensitive to antimycobacterial drugs. However, a large variety of mycobacterial mutants have been isolated and have been the subject of intensive study. Mutation can, and often does, result in an increased tolerance to inhibitory agents, particularly antibiotics. In some cases, mycobacteria alter genetic determinants encoding enzymes involved in the mechanism of action of the antibiotic rendering the drug ineffective. Multidrug resistant *M. tuberculosis* are those

bacteria that have become resistant to two or more antibiotics either by mutation or by acquisition of drug resistance determinants.

Though not wishing to be limited to any technical explanation, applicant believes that compounds of the invention inhibit the growth of slow growing mycobacteria through an autoinducer mechanism. Therefore an autoinducer can also be contemplated in the present invention.

An autoinducer is a small diffusible molecule which is produced and then secreted by a microorganism during metabolism and which then acts to increase or decrease the expression of the genes of the microorganism. Autoinducers may be synthesized by adding a fatty acid chain to a L-homoserine lactone (Figure 2). L-homoserine lactone is widespread in bacteria. Known autoinducers include L-homoserine lactone derivatives or modified L-homoserine lactones including N-acyl-L-homoserine lactones such as N-(3-oxohexanoyl)-L-homoserine lactone, N-(3-hydroxybutanoyl)-L-homoserine lactone, N-(3-oxododecanoyl)-L-homoserine lactone, N-(3-oxooctanoyl)-L-homoserine lactone, N-butanoyl-L-homoserine lactone, and N-hexanoyl-L-homoserine lactone. PCT Publication WO 92/18614 describes various members of this family of compounds, including optically active isomers thereof. Precursors of autoinducers can be used by the enzymes of a microorganism to produce autoinducers.

Bacteria use autoinducers to communicate intercellularly. These autoinducers function as quorum sensors in certain bacteria and, when present at sufficient concentrations, activate regulatory cascades leading to the transcription of genes

associated with high density growth. The first autoinducer reported was N-(3-oxohexanoyl) homoserine lactone (OHHL) from the marine symbiont bacterium *Photobacterium* (previously *Vibrio*)
5 *fischeri* (Eberhard et al., *Biochemistry*, 20:2444-2449, 1981). *P. fischeri* grows to densities of 10^{11} cfu/ml within the light organs of certain marine fish and squids. At these high densities, OHHL produced by the organism reaches a critical
10 concentration which leads to the activation of bioluminescence genes and light production (Eberhard, *J. Bacteriol.*, 109:1101-1105, 1972; Kaplan and Greenberg, *J. Bacteriol.*, 163:1210-1214, 1985). But when *P. fischeri* lives as a free organism
15 in sea water at low density (about 10^2 cfu/ml), the bacterium is not bioluminescent. Thus, the autoinduction system allows *P. fischeri* to discriminate between high-density and low-density environments, and to modulate bioluminescence
20 accordingly.

The molecular genetics of autoinducer biosynthesis and subsequent gene activation in *P. fischeri* is controlled by *luxI* and *luxR* genes. The gene product of *luxI* is responsible for the
25 synthesis of OHHL and LuxR is the receptor for the autoinducer. Once the autoinducer is bound, LuxR becomes a transcriptional activator that activates genes involved in bioluminescence production (Fuqua et al., *J. Bacteriol.*, 176:269-275, 1994). Similar
30 autoinduction systems that are controlled by *luxI/luxR* homologs have been identified from other species including *Pseudomonas aeruginosa*, *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Streptomyces* and others. Although the phenotypes

which the autoinducers activate may differ among species, the common theme for autoinduction is density-dependent gene activation. For example, in the opportunistic pathogen *P. aeruginosa*, *lasI* and 5 *lasR*, which are the homologs of *luxI* and *luxR*, respectively, regulate the production of exoenzymes which are important virulence factors (Gambello et al., *J. Bacteriol.*, 173:3000-3009, 1991; Gambello et al., *Infect. Immun.*, 61:1180-1184, 1993; Passador et 10 al., *Science*, 260:1127-1130, 1993; Toder et al., *Infect. Immun.*, 64:2062-2069, 1991).

The autoinducers produced by different bacteria are species-specific. Gram-positive bacterium *Streptomyces spp.* produce butyrolactones 15 rather than homoserine lactones as their autoinducers (Horinouchi and Beppu, *Annu. Rev. Microbiol.*, 46:377-398, 1992). Increasing concentrations of A-factor, a butyrolactone derivative (Figure 2), in stationary phase trigger 20 morphological and biochemical changes in *S. griseus* leading to sporulation and the production of streptomycin (Horinouchi et al., *J. Bacteriol.*, 171:1206-1210, 1989; Beppu, *Gene*, 115:159-165, 1992).

25 In addition to intercellular signaling with N-acyl-L-homoserine lactones or butyrolactones, other bacteria such as *Escherichia coli* are believed to use non-acylated L-homoserine lactone itself as an intracellular signaling molecule. In *E. coli* L-homoserine lactone accumulates in stationary phase 30 and is believed to stimulate the production of Rpos, a major regulatory protein for stationary phase survival. L-homoserine is the intermediate in the biosynthesis of methionine, threonine, and

isoleucine from aspartate, and homoserine lactone is the byproduct of the synthetic pathway (Figure 1).

According to a model proposed by Huisman and Kolter (Science, 265:537-539, 1994), L-homoserine lactone

- 5 could accumulate intracellularly in response to early amino acid deficiencies and thus provide an early warning signal for impending starvation.

Given the fact that autoinduction systems signaled by homoserine lactone or butyrolactone-like

- 10 inducers are common sensors for population control among diverse species of bacteria, the effects of exogenous homoserine on the proliferation of slow growing mycobacteria was studied. L-homoserine inhibits growth of *Mycobacterium smegmatis* - a non-

- 15 pathogenic rapid grower; the mechanism for growth inhibition is thought to be a blockade in glutamate uptake by L-homoserine (Sritharan et al., J. Gen. Microbiol., 133:2781-2785, 1987). L-homoserine is also toxic for slow growing mycobacteria in culture

- 20 by a bacteriostatic mechanism. However, its effect on glutamate uptake in *M. bovis* BCG cannot account for the growth inhibition observed.

In one embodiment, the invention compounds are employed to inhibit the growth of slow growing

- 25 mycobacteria including *M. bovis*, *M. tuberculosis*, and multidrug resistant *M. tuberculosis*. A mammal infected with slow growing mycobacteria can be treated with an effective amount of L-homoserine, L-

- 30 preferred embodiment an effective amount of these compounds is administered to a human infected with *M. tuberculosis*, *M. bovis* or multidrug resistant isolates of *M. tuberculosis*. This treatment kills or inhibits the growth of *M. tuberculosis*, *M. bovis* or

multidrug resistant *M. tuberculosis*. Other slow growing mycobacteria that may be affected by L-homoserine, L-homoserine lactone or a precursor thereof are described by Peterson (In: Rossman, M.D.

- 5 and MacGregor, R.R., eds. *Tuberculosis, Clinical Management and New Challenges*. McGraw-Hill, New York, pp. 359-372, 1995).

The invention may also be effective in treating disease caused by rapid growing non-tuberculous mycobacteria such as, for example, *M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. peregrinum*, and *M. marinum* (Wright and Wallance, In: Rossman, M.D. and MacGregor, R.R., eds. *Tuberculosis, Clinical Management and New Challenges*. McGraw-Hill, New York, pp. 373-389, 1995) by killing or inhibiting the growth of the relevant pathogen.

In some of the examples below, *M. bovis* BCG is sometimes used to demonstrate the invention because this mycobacteria is an art-recognized model 20 for the human pathogen *M. tuberculosis* (Jacobs and Bloom, In: Bloom B.R., ed. *Tuberculosis: Pathogenesis, Protection and Control*, ASM Press, Washington, DC, pp. 253-268, 1994).

The compounds are preferably formulated prior 25 to administration. Suitable pharmaceutical formulations are prepared by known procedures using well known and readily available ingredients (see Martin, E.W., *Remington's Pharmaceutical Sciences*, Mack Publishing, Easton, PA). The formulations 30 include those suitable for oral, parenteral (including subcutaneous, intradermal, intramuscular, intravenous, intrathecal, and intra-articular) and topical (including dermal, sublingual and intrabronchial) administration although the most

suitable route may depend upon, for example, the condition of the recipient. The formulation may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in
5 the art of pharmacy. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the
10 desired form.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, or tablets, each containing a predetermined amount of
15 the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid emulsion or a water-in-oil liquid emulsion. A preferred delivery vehicle for the treatment of intracellular infection is a liposome such as
20 described in, for example, U.S. Pat. Nos. 5,000,958 and 5,270,052). The active ingredient may also be presented as a bolus, electuary, or paste.

Formulations for parenteral administration include aqueous and non-aqueous sterile injection
25 solutions which may contain anti-oxidants, buffers, bacteriostats, and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions which may include suspending agents and
30 thickening agents may also be added.

Formulations for topical administration in the mouth, for example buccally or sublingually, include lozenges comprising the active ingredient in a flavored bases such as sucrose and acacia or

tragacanth, and pastilles comprising the active ingredient in a bases such as gelatin and glycerin, or sucrose and acacia. It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question.

The compounds of the invention may be administered orally or via injection at a dose of from about 2.5 mg/Kg body weight per day to about 2 g/Kg body weight per day. More preferably the dose would be about 25 mg/Kg body weight per day to about 200 mg/Kg body weight per day. The dose range for adult humans is generally from about 50 mg to about 300 g/day. One skilled in the art will recognize that the amount sufficient to inhibit the growth of slow growing mycobacteria varies depending upon an affected tissue size, age and body weight of the patient, and concentration of the compound in the formulation. Generally, the precise amount of compound to be administered as a therapeutic agent (e.g., amount in a dose, number of doses, timing of doses) will be determined on a case-by-case basis at the discretion of the attending physician. The extent of the mycobacterial infection, body weight, and age of the patient are also considerations.

Clinical specimens can be tested for the presence of slow growing mycobacteria including *M. tuberculosis* according to this invention. The clinical specimens can include samples obtained from biopsies, blood, and body discharge such as sputum, peritoneal fluid, pleural fluid, gastric content, spinal fluid, urine, and the like. Clinical

specimens can be inoculated into a cultivation medium. If the mycobacteria isolate grows in standard cultivation medium but not in cultivation medium when it contains L-homoserine, L-homoserine

- 5 lactone or precursors thereof, the presence of a slow growing mycobacterium is indicated and the presence of a rapid growing mycobacterium such as *M. smegmatis* or *M. vaccae* is eliminated.

In a cultivation medium, the minimal

- 10 inhibitory concentrations of L-homoserine or L-homoserine lactone for susceptible bacteria generally will range from about 22 to about 45 µg/ml in liquid cultivation medium and from about 25 to about 50 µg/ml in solid cultivation medium.

- 15 Resistant bacteria grow well at concentrations of L-homoserine and L-homoserine lactone in excess of about 200 µg/ml. To achieve differential inhibition, the preferred concentration of the added compound in the cultivation medium is from about 100 µg/ml to

- 20 about 200 µg/ml for either L-homoserine or L-homoserine lactone. The composition and the preparation of liquid and solid cultivation medium for mycobacteria are well known in the art and are commercially available. See chapter 7 "Current

- 25 Laboratory Methods for the Diagnosis of Tuberculosis" In: Bloom, B.R. ed. *Tuberculosis: Pathogenesis, Protection and Control*, ASM Press, Washington, DC, 1994 for a detailed description. The solid media can be, but is not limited to, egg-based

- 30 media (Lowenstein-Jensen Ogawa, and American Trudeau Society) and agar-based media (Middlebrook-Cohn 7H10 agar and 7H11 agar). The liquid media can be, but is not limited to, Middlebrook 7H12 broth and 7H9 broth. Slow growing mycobacteria usually grow at

37°C and pH between about 5.8 to about 8.0. The cultivation medium desirably contains some incorporated antibiotics which inhibit the growth of the non-mycobacterial contaminants. The composition 5 of the cultivation medium can affect the minimal inhibitory concentration.

Animal cells in culture or bacterial cells in culture are examples of samples that may be screened for mycobacteria. Cell and tissue cultures are terms 10 of art that refer to propagation in vitro of isolated animal cells, while bacterial culture refers to propagating bacteria in a liquid or solid medium in the presence or absence of host cells in vitro. Preferred host cells include mycobacterium-infectable cells, in particular cells of the 15 macrophage-phagocyte lineage. Such mycobacterium-infectable cells may be identified by exposing the putative mycobacterium-infectable cell to mycobacteria, and then utilizing the methods described in this application to detect the presence 20 of intracellular mycobacteria or their growth. Such cell lines are publicly available from the American Type Culture Collection (ATCC) and the NIGMS Human Genetic Mutant Cell Repository.

25 In another embodiment, the homoserine related compounds are employed to inhibit the growth of mycobacteria in infected macrophages, monocytes, histiocytes, Kupffer cells and microglia. These cells may be isolated from a human and cultured in vitro; in particular, alveolar macrophages may be obtained from bronchoalveolar lavage or peripheral blood macrophages.

Screening for other useful agents (e.g., an antibiotic known to the art, such as described in,

for example, U.S. Pat. Nos. 5,000,958 and 5,270,052) that have a therapeutic effect in combination with the invention compounds (preferably the effect is synergistic because separate and distinct aspects of mycobacterial metabolism are targeted), or for designing improved derivatives of the compounds which maximize activity and penetrability into human cells and/or minimize toxicity and side effects (e.g., acylated derivatives of L-homoserine lactone) are further embodiments of the invention. In a particularly preferred embodiment L-homoserine lactone can be added in a concentration of up to about 200 µg/ml to a human macrophage cell line infected with slow growing mycobacteria without toxic effect on the macrophage cells.

A mycobacterial gene or genetic program, as well as proteins encoded by such genes, may be regulated by homoserine or homoserine lactone. Mycobacterial cultures may be compared prior to and after treatment with homoserine or homoserine lactone. For example, if homoserine acted as an autoinducer in mycobacteria, gene transcription may be induced or repressed by addition of homoserine to the culture. Homoserine regulated genes may be identified by screening a subtractive cDNA library, or by differential screening of cDNA or genomic clone libraries of mycobacteria. Such regulated transcripts will be translated into regulated proteins, such proteins may be identified by comparing the pattern of proteins expressed prior to and after treatment with homoserine or homoserine lactone. For example, pre- and post-treatment cultures of mycobacteria may be pulsed with ³⁵S-amino acids, protein extracts may be made from whole cell

lysates or subcellular fractions, and regulated proteins may be identified by their increased or decreased signal intensity in two-dimensional gels ^{35}S -labeled proteins from pre- and post-treatment

5 cultures. Proteins of interest (i.e., labeled proteins which increase or decrease in abundance) may be isolated, N-terminal or internal peptide amino acid sequence may be determined, and the regulated gene of interest identified.

10 Homoserine or homoserine lactone regulated genes may also be identified by promoter trapping. A clone library of mycobacterial genomic DNA fragment inserted into a promoter probe vector (the general strategy is described in U.S. Pat. No. 4,725,535)

15 can be constructed to operably linked the DNA fragment with an indicator gene (e.g., lacZ, luxAB, xylE, firefly luciferase, the gene for green fluorescent protein or gfp, melC), such that a promoter contained in the DNA fragment may direct

20 the transcription of the indicator gene. A suitable indicator gene will be transcribed and produce a detectable indicator product under appropriate assay conditions. Individual clones of the library may be introduced into *M. tuberculosis* or the host cell,

25 and colonies replica plated under conditions either having or lacking homoserine or homoserine lactone. DNA fragments will be isolated form colonies which produce indicator product only when homoserine or homoserine lactone is present because they could contain homoserine-dependent promoters.

30 Alternatively, a construct containing the indicator gene but no operably linked promoter may be randomly integrated into the mycobacterial chromosome. Clones which contain integrations near homoserine-regulated

promoters may be identified after addition of homoserine by screening for the indicator product. Those integrations could mark the sites of homoserine-regulated promoters and isolating the mycobacterial genes associated with such promoters may also identify homoserine-regulated genes.

All books, articles, and patents cited in this specification are incorporated herein by reference in their entirety. In particular, the present invention was disclosed in U.S. Provisional Appn. No. 60/010,996, filed February 1, 1996, the entire contents of which are hereby incorporated by reference and relied upon.

The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

EXAMPLES

The strains of mycobacteria used were the *M. bovis* BCG (Pasteur strain, ATCC #5734), *M. tuberculosis* H37Rv (ATCC 27294), D-CS^R (ATCC 35826), *M. smegmatis* mc² 1-2C, a transformable variant of mc² 6 (Zhang et al., Mol. Microbiol., 5:381-391, 1991), and the A4 strain of *M. avium* (Cooksey et al., Antimicrob. Agents Chemother., 39:754-756, 1995). *M. tuberculosis* clinical isolates were obtained from Loayza Hospital (Lima, Peru). All mycobacterial strains were cultured in Middlebrook 7H9 broth or 7H10 agar (Difco) supplemented with 10% ADC (albumin dextrose complex), 50 µg/ml cycloheximide (Sigma), 0.2% glycerol and 0.05% Tween 80 (7H9 broth only). Nitrogen-depleted 7H9 medium was prepared by omitting monosodium glutamate and ammonium sulfate from the original formulation.

Example 1:

The BACTEC procedure for drug susceptibility testing of mycobacteria is based on the same principle employed in the conventional (agar) method except that a liquid medium is used. Instead of counting colonies, the growth is monitored radiometrically. Resistance is determined by comparing the rate of growth in the control and the vials containing test drug. When an antituberculous drug is added to the medium, suppression can be detected by a reduced daily growth index (GI) when compared to the control (Siddiqi, "Radiometric (BACTEC) tests for slowly growing mycobacteria." In: Isenberg, H.D. ed. *Clinical Microbiology Procedures Handbook*, Vol. 1. ASM Press, Washington, DC, 1992). Unlike conventional methods (agar dilution) which require 3 weeks, the BACTEC method results are usually reportable within 4 to 5 days. The growth of *M. bovis* BCG, Pasteur strain, ATCC 35734, *M. tb.* H37Rv, ATCC 25618, *M. tb.* JHH strain 9453 (a multidrug resistant strain resistant to isoniazid at 0.1 µg/ml, resistant to rifampin at 1.0 µg/ml, resistant to ethambutol at 2.5 µg/ml, and resistant to streptomycin at 2.0 µg/ml), *M. smegmatis* and *M. vaccae* was tested.

Inoculum from growth on solid medium such as Middlebrook 7H10/7H11 or LJ slants was added to 2 ml of the special diluting fluid (Siddiqi, "Radiometric (BACTEC) tests for slowly growing mycobacteria." In: Isenberg, H.D. ed. *Clinical Microbiology Procedures Handbook*, Vol. 1. ASM Press, Washington, DC, 1992) containing glass beads. Then, the special diluting fluid was vortexed until the suspension is well dispersed with as few clumps as possible. The

suspension was showed to stand for at least 30 minutes to allow mycobacterial clumps to settle. Then the homogeneous supernatant was transferred to a sterile tube and the turbidity was adjusted to

5 match a 0.5 McFarland standard. One-tenth ml of the inoculum prepared in this manner was added to Bactec 12B culture bottles (Becton Dickinson, Sparks, MD) containing L-homoserine or L-homoserine lactone at various concentrations ranging from 0 to 180 µg/ml.

10 Growth index (GI) values were measured at 24 hour intervals using a Bactec 460 TB radiometric growth detection machine (Becton Dickinson, Sparks, MD). The difference in the GI values from the previous day is designated as ΔGI. Negative or zero ΔGI

15 values indicate no growth, while positive ΔGI values indicate an increase in growth. An isolate was determined to be susceptible to a particular concentration of L-homoserine or L-homoserine lactone if the ΔGI for the test sample was less than

20 the ΔGI for the untreated (drug concentration = 0) control, provided that the untreated control reached an absolute GI of 30 or more. If the ΔGI for the test sample exceeds or is similar to that for the untreated control, the isolate was resistant to that

25 particular concentration of L-homoserine or L-homoserine lactone. It was shown that L-homoserine lactone inhibited the growth of *M. bovis* BCG, Pasteur strain, ATCC 35734, *M. tb.* H37Rv, ATCC 25618, *M. tb.* JHH strain 9453, resistant to INH,

30 RIF, EMB, STR, with a minimum inhibitory concentration (MIC) of 22-45 µg/ml. In contrast, they have no effect upon *M. smegmatis* and *M. vaccae* with the MIC being higher than 200 µg/ml.

Similar results have been obtained for L-homoserine lactone with *M. bovis* BCG and *M. smegmatis* using the agar dilution method (Kent and Kubica, *Public Health Mycobacteriology: A Guide for the Level III Laboratory*. U.S. Dept. of Health and Human Services, Atlanta, GA, 1985). Cultures of *M. bovis* BCG or *M. smegmatis* grown in 7H9 supplemented with ADC (Kent and Kubica, *ibid.*) and cycloheximide (50 µg/ml) were first adjusted to a McFarland standard of 1, and then diluted to 10⁻² and 10⁻⁴ for *M. bovis* BCG or 10⁻³ and 10⁻⁵ for *M. smegmatis*. Quadrant plates prepared with 7H10 medium containing different concentrations of homoserine lactone were inoculated with diluted suspensions, 0.1 ml per each quadrant and one suspension per plate. One of the four quadrants in each plate functions as control medium without L-homoserine lactone. The inoculum was spread evenly in each quadrant using a sterile loop. The plates were air-dried and incubated at 37°C for 3 weeks for *M. bovis* BCG, *M. avium*, or *M. tuberculosis* strains; or 5 days for *M. smegmatis*. For *M. bovis* BCG, colonies were observed in the quadrants with 0 or 25 µg/ml L-homoserine lactone, but not in the quadrants with 50 or 100 µg/ml L-homoserine lactone; whereas, for *M. smegmatis* was observed in the quadrants with L-homoserine up to 200 µg/ml.

To determine how susceptible various mycobacteria are to homoserine and homoserine lactone, several commonly used laboratory strains were selected including rapid and slow growers, and pathogenic and nonpathogenic mycobacteria. Using the agar dilution method these mycobacteria were tested against various concentrations of D-homoserine, L-

homoserine, and L-homoserine lactone. The minimal inhibitory concentration (MIC) is the concentration which inhibited the bacterial colony forming units by more than 75% compared to the controls. The MIC 5 for each strain of mycobacteria used are listed in Table 1. *M. bovis* BCG was susceptible to L-homoserine and L-homoserine lactone with MIC varying between 25-50 µg/ml. D-homoserine had little effect on *M. bovis* BCG growth on 7H10 agar plates, 10 indicating that only the L-isomer is active as a growth inhibitor. *M. avium*, another slow growing mycobacterium which is the opportunistic pathogen in immunocompromised individuals, was also sensitive to 50 µg/ml of L-homoserine and L-homoserine lactone. 15 In contrast, the rapid growing nonpathogenic mycobacterium *M. smegmatis*, was resistant to L-homoserine and L-homoserine lactone. A commonly used laboratory strain of *M. tuberculosis*, H37Rv, although it is slow growing and pathogenic, was able 20 to grow in the presence of 100 µg/ml of L-homoserine and L-homoserine lactone. Since H37Rv has been adapted in laboratories for almost a century, it may differ from clinical isolates of *M. tuberculosis* in terms of its physiological behavior.

25 Table 1: Susceptibility of mycobacteria to homoserine (HS), homoserine lactone (HSL), or cycloserine (CS).

^a determined by dilution in agar culture.

^b ND = not done.

Table 1
MIC (μ g/ml)^a

	D-HS	L-HS	L-HSL	D-CS
<i>M. bovis</i> BCG	>200	25-50	25-50	40
<i>M. smegmatis</i> mc ² 100	>200	>200	>200	ND ^b
<i>M. avium</i>	>200	50	50	<12.5
<i>M. tuberculosis</i> H37Rv	>100	>100	>100	25
<i>M. tuberculosis</i> D-CS ^R	>100	>100	>100	50
<i>M. bovis</i> BCG HS ^R 1	>200	>100	>100	40

In view of the somewhat similar chemical structure of homoserine lactone and cycloserine (see Figure 2), it was important to address whether L-homoserine or L-homoserine lactone might mimic D-cycloserine to act as an inhibitor of peptidoglycan biosynthesis (see U.S. Pat. No. 5,260,324 for a discussion of unwanted side-effects associated with cycloserine treatment). To address this question, the MIC to D-cycloserine, a second line anti-
5 mycobacterial agent, was determined for several slow growing mycobacteria including a D-cycloserine resistant H37Rv strain and an L-homoserine resistant *M. bovis* BCG. As shown in Table 1, *M. bovis* BCG-HS^R 1
10 was sensitive to D-cycloserine with the same MIC value of 40 µg/ml as its parental *M. bovis* BCG strain, indicating there is no cross resistance between L-homoserine and D-cycloserine. This
15 observation implies that the mechanism of growth inhibition by L-homoserine against slow growing
20 mycobacteria differs from the mechanism of action of D-cycloserine.

To evaluate the activity of L-homoserine and L-homoserine lactone in recent clinical isolates of *M. tuberculosis*, susceptibility of eight strains collected in 1995 in Lima, Peru was determined. As seen in Table 2, there is a spectrum of susceptibility levels to the L-homoserine in these clinical isolates. Four of them had an MIC of 50 µg/ml, two were very sensitive to L-homoserine with an MIC less than 25 µg/ml, and two were resistant to 100 µg/ml of L-homoserine. The sensitivity of these isolates to L-homoserine was independent of their susceptibility profiles to other commonly used anti-mycobacterial agents. For example, strain TBL002EP2

was susceptible to all five antibiotics tested but was relatively resistant to L-homoserine; whereas TBL019EP21, an MDR isolate, was susceptible to 50 µg/ml of L-homoserine. Our results suggest that 5 sensitivity to L-homoserine varies among *M. tuberculosis* strains.

Table 2: Susceptibility of clinical isolates of *M. tuberculosis* to L-homoserine (L-HS), isoniazid (INH), streptomycin (SM), ethambutol (EMB), rifampin 10 (RA), and pyrazinamide (PZA).

^a determined by dilution in agar culture.

^b determined by BATEC at the University Hospital (Albuquerque, NM).

^c resistant to low concentration of SM (2 µg/ml).

15 ^d border level resistance.

Table 2
MIC ($\mu\text{g/ml}$)^a **Susceptibility to common anti-**
mycobacterial drugs^b

	L-HS	INH	SM	EMB	RA	PAZ
TBL002EP2	100	S	S	S	S	S
TBL019EP21	50	R	R ^c	R	R	S
TBL024EP28	<25	S	R ^c	S	S	S
TBL054EP66	50	R	S	R	S	R
TBL057EP70	50	R	S	S	R	BR ^d
TBL059EP73	<25	S	S	S	S	S
TBL141EP205	100	S	S	S	S	S
TBL194EP281	50	S	S	S	S	S

Example 2:

A ^3H -uracil incorporation assay was used to monitor the viability of *M. bovis* BCG exposed to different concentrations of L-homoserine lactone.

5 Log-phase growing *M. bovis* BCG in 7H9 ($\text{OD}_{600} = 0.5$ to 0.6) was transferred to 24-well tissue culture plate, 1 ml per well. Homoserine lactone was added to final concentrations of 50, 100, and 200 $\mu\text{g}/\text{ml}$. The cultures were incubated at 37°C in 5% CO_2 for 7
10 days. On each day after homoserine lactone treatment, aliquots of 5 μl were removed from each well in triplicate and transferred to wells in a 96-well plate containing 195 μl 7H9 medium. The suspensions were then mixed with 50 μl of 7H9 medium
15 containing ^3H -uracil (20 $\mu\text{Ci}/\text{ml}$). After 6 hours labeling at 37°C in 5% CO_2 , the bacilli were harvested onto a glass fiber filter, which was then washed with water, air-dried, and radioactivity on the filter was detected with an automatic
20 scintillation counter. It is clear that *M. bovis* BCG treated with 50 $\mu\text{g}/\text{ml}$ of L-homoserine lactone (a dose known to inhibit growth) continue to utilize uracil and hence are metabolically active (Figure 3). These results show that L-homoserine lactone is
25 bacteriostatic rather than bacteriocidal.

Example 3:

Uracil, methionine, and acetate uptake was measured with homoserine treated *M. bovis* BCG.

Dispersed *M. bovis* BCG suspensions in 7H9 with an
30 optical density of 1 McFarland standard (approximately 10^8 bacilli) were diluted to 10^{-2} , and 0.2 ml of each suspension were aliquoted in 96-well plates. Stock solutions of D-homoserine, L-

homoserine, or D-cycloserine (10 mg/ml prepared in PBS) were added to the *M. bovis* BCG cultures and the final concentrations of these compounds were 25, 50, 100 and 200 µg/ml. Control cultures were treated
5 with PBS only. Multiple samples were set up for labeling with different molecules. *M. bovis* BCG bacilli were continuously cultured in the presence or absence of the above compounds at 37°C under 5% CO₂. At various time points, cultured bacilli were
10 labeled with 1 µCi per well (50 µl of 20 µCi/ml solution) of ³H-uracil (specific activity of 49 Ci/mmol, Amersham), ³H-methionine (specific activity of 197 mCi/mmol, DuPont NEN), or ³H-acetate (specific activity of 100 mCi/mol, DuPont NEN). After a 6 hr
15 incubation, *M. bovis* BCG in the labeled cultures were harvested onto glass fiber filters (Packard) using a Filtermate 196 harvester (Packard). The filters were then washed six times with water and air dried. The radioactivity associated with each
20 sample on filters was detected using a Matrix™ 96 Direct Beta Counter (Packard).

Homoserine reduces utilization of precursors for nucleic acid, protein, or lipid biosynthesis. The uptake of radiolabeled uracil has been used as a
25 quantitative measure for estimating mycobacterial abundance (Flesch and Kaufmann, Infect. Immun., 56:1464-1469, 1988). This technique was used to evaluate the effects of homoserine on *M. bovis* BCG growth. *M. bovis* BCG was labeled with ³H-uracil in
30 liquid culture medium containing various concentrations of D-homoserine or L-homoserine. As shown in Figure 4 A and B, while the amount of cell-associated uracil in untreated or D-homoserine treated *M. bovis* BCG cultures increased continuously

over a 7 day period, the ability of *M. bovis* BCG to incorporate uracil was significantly inhibited by L-homoserine. The levels of inhibition by L-homoserine were similar to those obtained with 50 µg/ml D-cycloserine, a known mycobactericidal agent.

L-homoserine treated *M. bovis* BCG was also evaluated for specific reductions in lipid or protein biosynthesis by monitoring the incorporation of ^3H -acetate and ^3H -methionine, respectively. As shown in Figure 4 C-F, treatment with L-homoserine drastically reduced the incorporation of both ^3H -acetate and ^3H -methionine. Thus, L-homoserine appears to have global effects on *M. bovis* BCG metabolism in vitro, and these incorporation studies have not demonstrated specific reductions in nucleic acid, lipid, or protein biosynthesis by treatment with this compound.

The uptake studies also showed that D-homoserine was able to reduce the amount of uracil, acetate, and methionine taken up by *M. bovis* BCG as compared to untreated controls (Figure 4 A, C and E). However, D-homoserine was considerably less effective in blocking uptake than L-homoserine (Figure 4 B, D and F), as the levels of cell-associated radioactivities were gradually increasing over the 7 day period in the D-homoserine treated cultures whereas no increases were detected in the L-homoserine treated cultures.

Example 4:

To determine whether the effect of L-homoserine lactone on *M. bovis* BCG growth is bactericidal or bacteriostatic, after *M. bovis* BCG was treated with homoserine lactone for 7 days, the

cultures were diluted 1000-fold in homoserine lactone-free 7H9 medium. Two hundred μ l of each dilution were pipetted, in triplicate, into a 96-well plate (a total of three plates were prepared).

- 5 One plate was labeled immediately with 3 H-uracil as described above, which gives the basal level of transcription after homoserine lactone treatment.
- 10 The other two plates were labeled with 3 H-uracil for a one or two week incubation, at 37°C and 5% CO₂. The amount of radioactivity incorporated by bacteria grown in these plates was compared to the basal level before homoserine lactone treatment. These results demonstrated that 3 H-uracil utilization resumes after L-homoserine lactone treatment; the nature of inhibition is also examined in Example 5 and Figure 5.

Example 5:

- Viability was evaluated in agar culture. To determine whether L-homoserine is bactericidal or bacteriostatic, *M. bovis* BCG (approximately 10⁶ cfu/ml) in 2 ml cultures was first treated with 200 μ g/ml of D-homoserine, L-homoserine, or L-homoserine lactone; 0.1 μ g/ml of isoniazid (bactericidal); 10 μ g/ml of ethambutol (bacteriostatic); or with PBS (untreated control) in a 94-well plate at 37°C and 5% CO₂, for five days. Bacilli in each culture were then resuspended in fresh medium, and 0.1 ml of undiluted or diluted suspensions were inoculated onto antibiotic-free Middlebrook 7H 10 agar plates.
- 20 The number of colonies on each plate were counted after 3 weeks incubation at 37°C in a humidified 5% CO₂ incubator. The colony forming units (cfu) of the initial *M. bovis* BCG culture were determined by

plating a series of dilutions of this culture onto 7H10 agar plates in duplicate. The cfu's obtained from day 0 and day 5 after treatment were compared.

Growth inhibition of *M. bovis* BCG by L-homoserine appears to be bacteriostatic. An additional aspect of the mechanism of action of L-homoserine on slow growing mycobacteria which was evaluated was whether it is bacteriostatic or bacteriocidal. Since tubercle bacilli are capable of entering quiescent states during infection (Gedde-Dahl, Am. J. Hyg., 56:139-214, 1952) and in vitro (Wayne and Sramek, Infect. Immun., 64:2062-2069, 1996), this question was approached by comparing the ability of *M. bovis* BCG to survive treatment with L-homoserine as compared with a known bacteriocidal drug, isoniazid, and a known bacteriostatic agent, ethambutol. Surviving colony forming units was measured following a 5-day exposure of *M. bovis* BCG to L-homoserine, isoniazid, or ethambutol. As may be seen in Figure 5, both the untreated culture and that treated with 200 µg/ml of D-homoserine proliferated by at least a factor of 10 over the 5 day period as measured by colony forming units. In contrast, the numbers of live *M. bovis* BCG recovered from the 5 day cultures treated with 200 µg/ml of L-homoserine or 200 µg/ml of L-homoserine lactone (each at least 4-fold above MIC) were both 9 orders of magnitude less than the number of live *M. bovis* BCG in the starting culture. This level of recovery was similar to that observed for *M. bovis* BCG culture treated with 10 µg/ml of ethambutol (at least 2.5-fold above MIC) and was about 10 times higher than the live *M. bovis* BCG present in the culture treated with 0.1 µg/ml of isoniazid (at

least 4-fold above MIC). Thus, the inhibitory effect of L-homoserine on *M. bovis* BCG growth is more like the bacteriostatic effect of ethambutol.

Example 6:

- 5 Glutamate uptake was assayed with homoserine treated *M. bovis* BCG. Homoserine sensitive and homoserine resistant *M. bovis* BCG strains, which were selected for growth on high concentrations of homoserine and homoserine lactone, were cultured in
- 10 7H9 medium in a 37°C horizontal shaker. Bacteria were harvested in the late log phase by centrifugation at 3000 rpm for 5 m. The cell pellet was washed twice with PBS and resuspended in nitrogen-depleted 7H9 broth to its original volume.
- 15 After an overnight incubation, the bacteria were again harvested and the pellet was resuspended in fresh nitrogen-depleted 7H9 medium. One milliliter portions of the *M. bovis* BCG suspension were transferred to 1.5 ml microfuge tubes and cultured
- 20 in the presence of PBS or 200 µg/ml of D-homoserine, L-homoserine, or L-homoserine lactone at 37°C with shaking for 2 hr. *M. bovis* BCG were then labeled with 10 µl of ³H-glutamic acid (specific activity of 50 mCi/mmol, Amersham). After 30 min incubation the
- 25 glutamate uptake reaction was terminated by immediately chilling the reaction tubes on ice. Cells were spun down quickly in a microcentrifuge at 4°C and washed 3 times with ice-chilled glutamate-containing 7H9 medium. After a final wash, the *M.*
- 30 *bovis* BCG pellet was resuspended in 2 ml of liquid scintillation fluid and counted in a scintillation counter for 1 min. Heat-killed (30 min at 80°C) *M. bovis* BCG was used as control for the non-specific

attachment of ³H-glutamate to these bacilli. The dpms from killed *M. bovis* BCG samples were subtracted from the dpms of live *M. bovis* BCG samples. The amount of glutamate taken up by homoserine treated 5 *M. bovis* BCG was presented as the percentage of the untreated controls.

Specific glutamate-uptake inhibition may not be the apparent mechanism for L-homoserine-induced growth inhibition in *M. bovis* BCG. Sritharan et al. 10 (J. Gen. Microbiol., 133:2781-2785, 1987) have shown L-homoserine specifically inhibited glutamate transport in *M. smegmatis*, and addition of L-homoserine inhibited the growth of this saprophytic mycobacterium with an MIC of about 300 µg/ml. To 15 determine whether L-homoserine had similar effect on glutamate transport in *M. bovis* BCG, glutamate uptake assays were performed with *M. bovis* BCG in the presence of L-homoserine. As shown in Figure 6 A, glutamate uptake was inhibited by L-homoserine only at 100 µg/ml or greater. The inhibition was 20 specific for L-homoserine since D-homoserine (Figure 6 A) and L-homoserine lactone (not shown) did not inhibit glutamate uptake in *M. bovis* BCG. Although the degree of inhibition varied among experiments, 25 the average level of glutamate uptake inhibition with 200 µg/ml of L-homoserine was 58% as compared to the PBS treated *M. bovis* BCG cultures (Figure 6 B). It is unlikely that partial glutamate uptake blockade accounts for the complete growth inhibition 30 by this concentration of L-homoserine on *M. bovis* BCG.

To explore the possibility that the inhibitory effect that L-homoserine had on glutamate transport in *M. bovis* BCG might account for the

growth inhibition induced by this compound, the inhibition of glutamate uptake by L-homoserine was tested in two L-homoserine resistant *M. bovis* BCG mutants, HSR1 and HSR6. These spontaneous mutants
5 were selected on 7H10 agar containing 200 µg/ml of L-homoserine. As may be seen in Figure 6 B, there was no significant difference between the glutamate uptake inhibition by L-homoserine in the sensitive wild type *M. bovis* BCG strain compared with the two
10 L-homoserine resistant *M. bovis* BCG mutants. Thus L-homoserine partially blocks glutamate uptake in both drug sensitive and drug resistant strains. Moreover, the concentrations of L-homoserine at which glutamate transport was inhibited were higher (200
15 µg/ml) than the MIC of L-homoserine for sensitive *M. bovis* BCG (25-50 µg/ml). These observations indicate that the inhibitory effect of L-homoserine on glutamate transport cannot completely account for the inhibitory effect of L-homoserine on growth in
20 *M. bovis* BCG.

Example 7:

The human macrophage cell line, U937 (ATCC CRL 1593; Sundstrom and Nilsson, Intl. J. Cancer, 17:565-577, 1976), was used for the toxicity assay.
25 U937 cells (human promonocytic cell line), 5×10^4 cells per well, were treated with L-homoserine lactone at a concentration of 0, 7.5, 15, 30, 60, 120, 240, or 480 µg/ml in 96-well plates.
Following a 24 hour or 72 hour incubation,
30 U937 cells were labeled with ^3H -thymidine for 6 hours. The labeled cells were harvested on a glass fiber filter, lysed and washed with water. After the filter was dry, the radioactivity was measured by

scintillation counting and the amounts of tritium incorporation for the treated groups were compared to the untreated controls at each time point. These results showed that L-homoserine is non-toxic for 5 the human macrophage cell line U937 at levels that are bacteriostatic for mycobacteria.

The principles, preferred embodiments and modes of operation of the present invention have been described in the foregoing specification. The 10 invention which is intended to be protected herein, however, is not to be construed as limited to the particular forms disclosed, since they are to be regarded as illustrative rather than restrictive. Variations and changes may be made by those skilled 15 in the art without departing from the spirit of the invention.

CLAIMS:

1. A method for inhibiting growth of a mycobacterium, comprising: administering to a mammal suspected of being infected with said mycobacterium,
5 a growth inhibiting amount of L-homoserine lactone or L-homoserine or a precursor thereof.
2. The method of claim 1, wherein said mycobacterium is a slow growing mycobacterium.
3. The method of claim 2, wherein said slow
10 growing mycobacterium is a species selected from the group consisting of *M. bovis*, *M. tuberculosis*, and multidrug resistant *M. tuberculosis*.
4. The method of claim 2, wherein said slow
15 growing mycobacterium is a tuberculous mycobacterium or a non-tuberculous mycobacterium.
5. The method of claim 1, wherein said mycobacterium is a tuberculous mycobacterium selected from the group consisting of *M. africanum*,
M. bovis, *M. microti*, and *M. tuberculosis*.
- 20 6. The method of claim 1, wherein said mycobacterium is a non-tuberculous mycobacterium selected from the group consisting of *M. avium*, *M. intracellulare*, *M. kansasii*, and *M. leprae*.
- 25 7. The method of claim 1, wherein said precursor is L-homoserine phosphate.

8. A composition for use in the method of
claim 1, wherein the composition comprises:

- (a) L-homoserine or L-homoserine lactone or
a precursor thereof, and
5 (b) an anti-mycobacterial agent.

9. The composition of claim 8, wherein the
anti-mycobacterial agent is selected from the group
consisting of D-cycloserine, ethambutol, isoniazid,
pyrazinamide, rifampin, and streptomycin.

10 10. A method for inhibiting growth of a slow
growing mycobacterium, comprising: administering an
effective amount of L-homoserine lactone or L-
homoserine or a precursor thereof to inhibit the
growth of said mycobacterium.

15 11. The method of claim 10, wherein said
mycobacterium is a tuberculous mycobacterium or a
non-tuberculous mycobacteria.

20 12. The method of claim 10, wherein said
mycobacterium is a species selected from the group
consisting *M. africanum*, *M. avium*, *M. bovis*, *M.*
intracellulare, *M. kansasii*, *M. leprae*, *M. microti*,
and *M. tuberculosis*.

13. The method of claim 10, wherein said
precursor is L-homoserine phosphate.

25 14. A method of identifying a slow growing
mycobacterium, comprising: comparing growth of a
clinical specimen in a first cultivation medium with
growth in a second cultivation medium comprising an

inhibiting molecule selected from the group consisting of L-homoserine, L-homoserine lactone, and a precursor thereof, wherein growth in said first cultivation medium but not in said second cultivation medium indicates the presence of the slow growing mycobacteria.

15. The method of claim 14 wherein said precursor is L-homoserine phosphate.

16. The method of claim 14 wherein said slow growing mycobacteria is a species selected from the group consisting of *M. africanum*, *M. avium*, *M. bovis*, *M. intracellulare*, *M. kansasii*, *M. leprae*, *M. microti*, and *M. tuberculosis*.

17. A cell line, wherein said cell line comprises a slow growing mycobacterium resistant to L-homoserine.

18. The cell line of claim 17, wherein said slow growing mycobacterium is a species selected from the group consisting of *M. africanum*, *M. avium*, *M. bovis*, *M. intracellulare*, *M. kansasii*, *M. leprae*, *M. microti*, and *M. tuberculosis*.

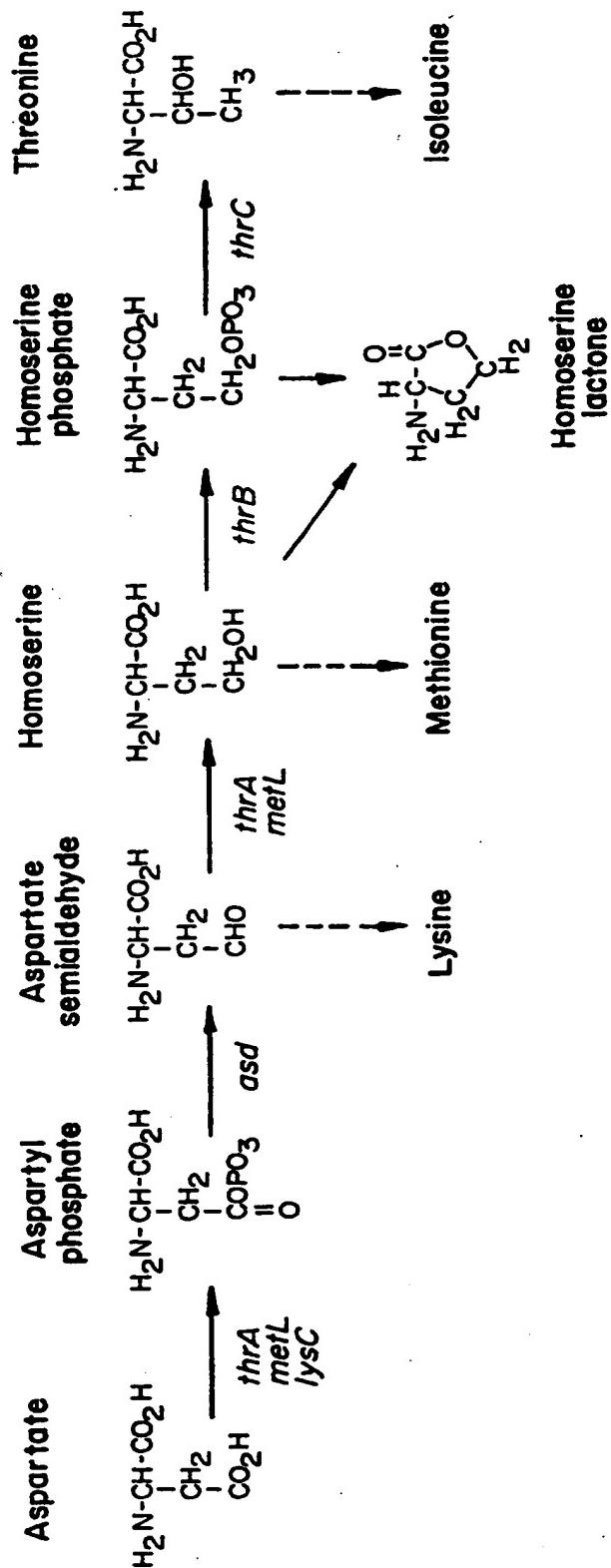
19. The cell line of claim 17, wherein said slow growing mycobacterium resistant to 200 µg/ml L-homoserine.

25. 20. A method of identifying a gene in a mycobacterium regulated by an anti-mycobacterial agent, comprising: detecting induction or repression of expression of said gene in response to culturing

said mycobacterium with L-homoserine or L-homoserine lactone or a precursor thereof.

1/6

FIG. 1



SUBSTITUTE SHEET (RULE 26)

2/6

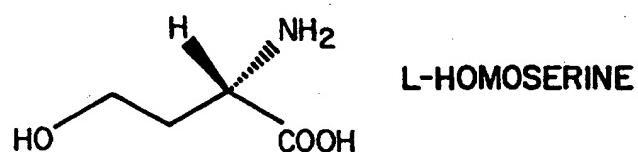
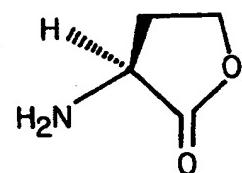
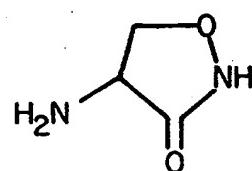
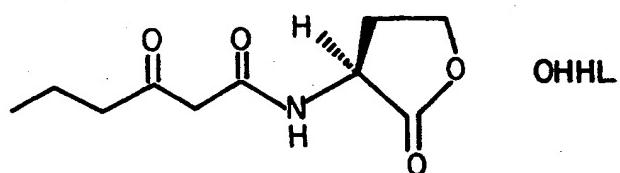
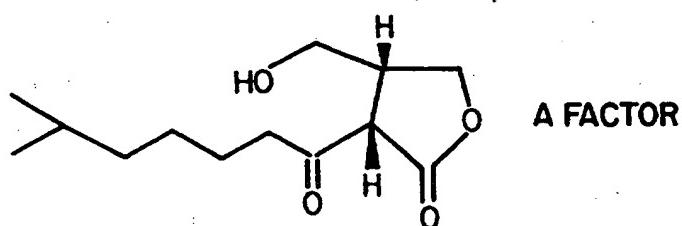
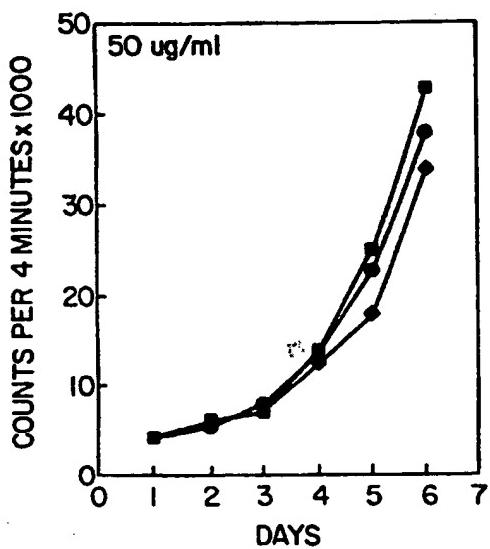
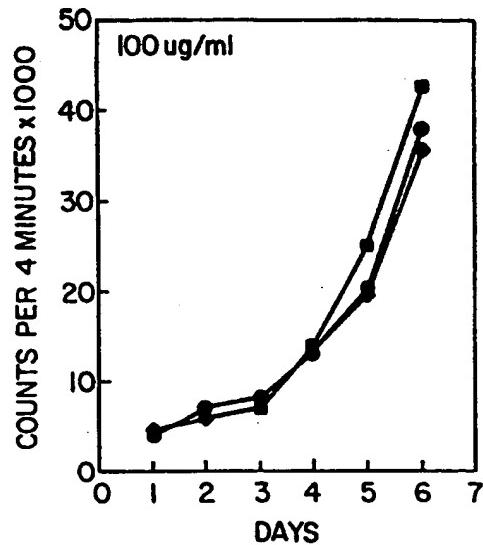
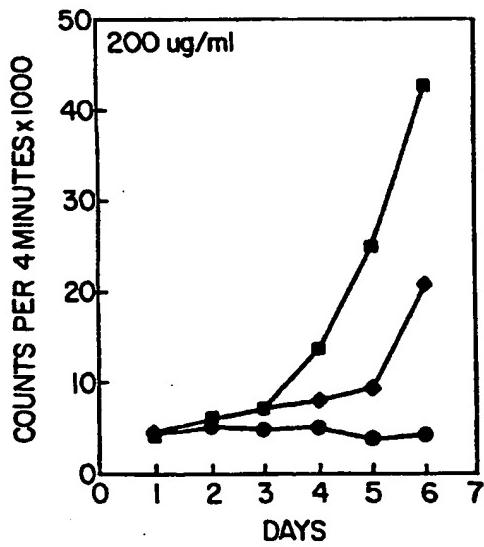
FIG. 2**L-HOMOSERINE****L-HOMOSERINE
LACTONE****D-CYCLOSERINE****OHHL****A FACTOR****SUBSTITUTE SHEET (RULE 26)**

FIG. 3A**FIG. 3B****FIG. 3C****SUBSTITUTE SHEET (RULE 26)**

4/6

FIG. 4A

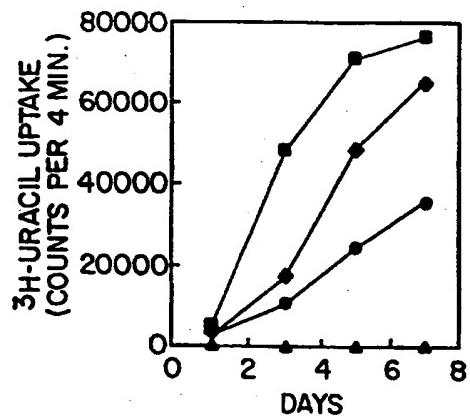


FIG. 4B

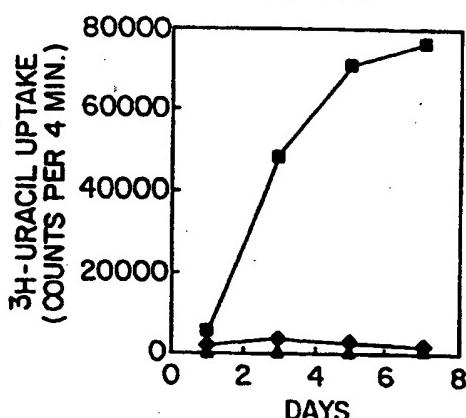


FIG. 4C

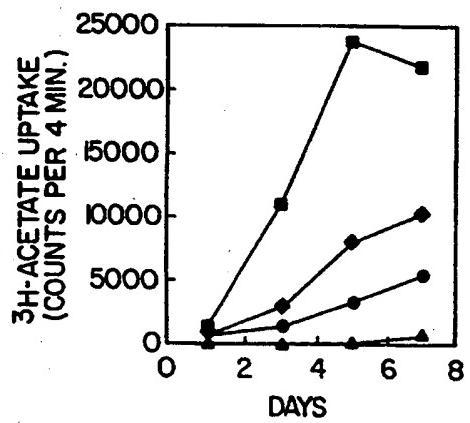


FIG. 4D

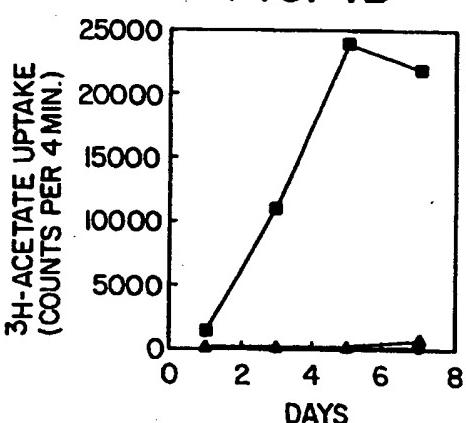


FIG. 4E

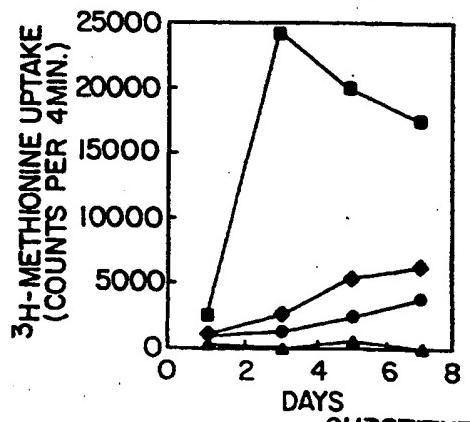
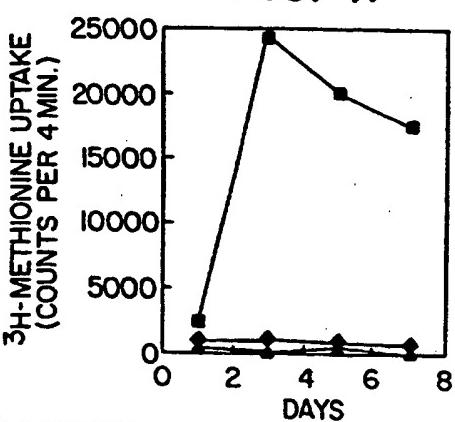
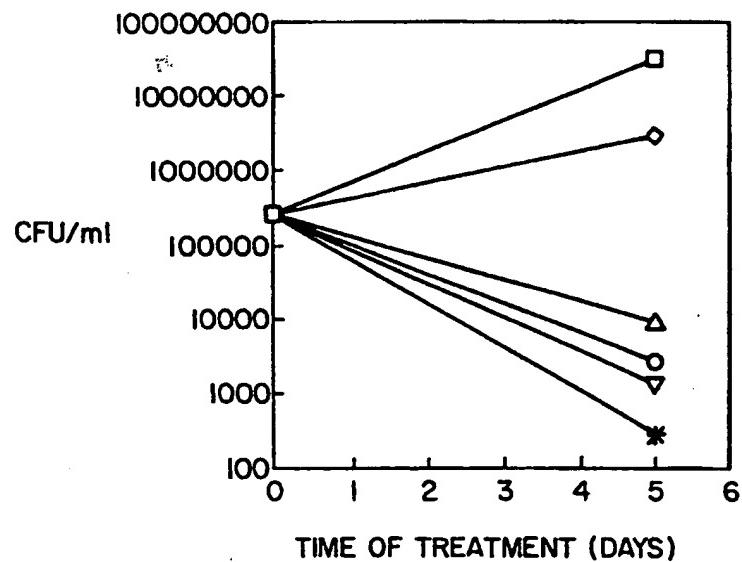


FIG. 4F

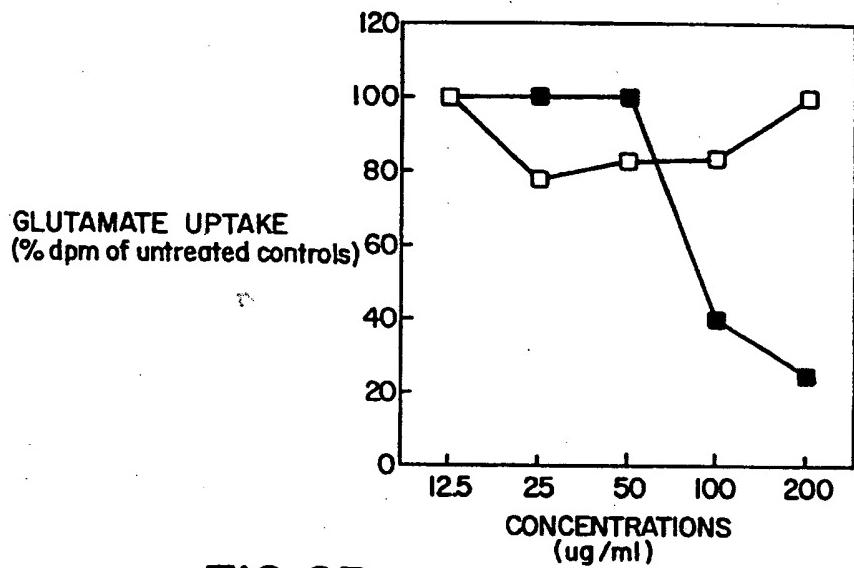
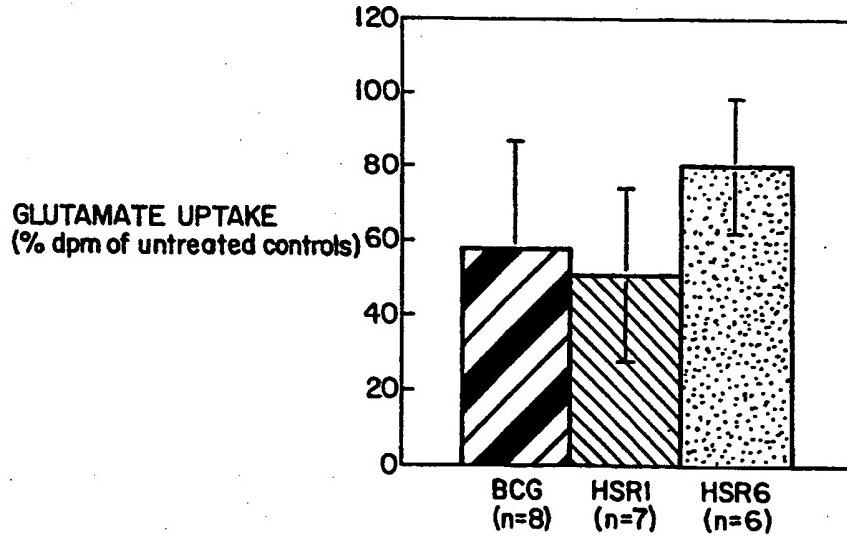


SUBSTITUTE SHEET (RULE 26)

5/6

FIG. 5**SUBSTITUTE SHEET (RULE 26)**

- 6/6

FIG. 6A**FIG. 6B****SUBSTITUTE SHEET (RULE 26)**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US97/01241

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 31/365; C07D 307/33

- US CL : 514/9; 435/41

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/9; 435/41

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FEMS Microbiology Letters, Volume 140, issued 1996, Stead et al., "Induction of phenazine biosynthesis in cultures of <i>Pseudomonas Aeruginosa</i> by L-N-(3-oxohexanoyl)homoserine lactone", pages 15-22, see pages 15-22.	1-20
A	US,A,3,655,510 (TANAKA ET AL) 11 April 1972, see entire document	1-20

Further documents are listed in the continuation of Box C.

See patent family annex.

- Special categories of cited documents:
- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed
- *R* document member of the same patent family

Date of the actual completion of the international search

10 JUNE 1997

Date of mailing of the international search report

24 JUN 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

BENET PRICKRIL

Telephone No. (703) 308-0196

THIS PAGE BLANK (USPTO)